World Health Organization

Pyrosequencing Analysis Protocol for the Detection of the Substitution at Residue 275 in the Neuraminidase of 2009 Pandemic H1N1 Viruses Using the PyroMark™ Q24 Platform

November 2012

The WHO Collaborating Centre for the Surveillance, Epidemiology and Control of Influenza at the Centers for Disease Control and Prevention (CDC) Atlanta, United States of America, has made available this protocol for antiviral susceptibility testing of influenza A(H1N1)pdm09.

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Centers for Disease Control
and Prevention (CDC)
Atlanta, GA 30333

Pyrosequencing Analysis Protocol for the Detection of the Substitution at Residue 275 in the Neuraminidase of 2009 Pandemic H1N1 Viruses Using the PyroMark™ Q24 Platform

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I. Introduction

For the prophylaxis and treatment of influenza A virus infections there are currently two classes of drugs that are available, M2 blockers (adamantanes) and neuraminidase inhibitors (NAIs). The emergence of influenza viruses resistant to these antiviral drugs have yielded a need for the extensive surveillance of molecular markers associated with antiviral resistance.

A variety of methods are available for the monitoring of molecular markers associated with drug resistance. Sanger sequencing, high-resolution melting curve and real-time reverse-transcriptase PCR have been employed for the analysis of these molecular markers within the influenza virus genome. Although these methods are used for the analysis of influenza virus genomic variation, they have limitations. For example, real-time reverse-transcriptase PCR is dependent on the detection of specific mutations and is unable to detect additional mutations. The Sanger sequencing method has the ability to identify mutations, but it is not suitable for quantitative single nucleotide polymorphism (SNP) analysis. Sanger sequencing is also laborious, time consuming and it can be difficult to interpret the sequence chromatograms from samples containing multiple virus variants (quasispecies).

Pyrosequencing technology differs from other sequencing technologies; during DNA synthesis, it generates a sequence in real-time. Nucleotide sequences are determined by the sequential addition and subsequent incorporation of nucleotides, which trigger a cascade of enzymatic reactions resulting in the release and detection of light. The amount of light signal produced is proportional to the amount of pyrophosphate that is released upon nucleotide incorporation, making it a quantitative analysis.

Accurate quantitative analysis, together with other characteristics, makes the pyrosequencing method a desirable for the detection of influenza molecular markers associated with resistance to antiviral drugs. Pyrosequencing technology on the Biotage/QIAGEN platform is better suited for the analysis of short sequences, sequencing up to 100 nucleotides accurately. The quantitative analysis of nucleotide incorporation allows for an accurate SNP analysis, enabling the detection of minor variants present at 10% or lower. In one pyrosequencing run, it is possible to analyze up to 96 samples (PyroMark Q96), which is valuable in the high-throughput screening for molecular markers associated with drug resistance. This method is also less laborious than the conventional Sanger sequencing and preparation of the pyrosequencing reactions with RT-PCR template along with the pyrosequencing run may be completed within 1.5-2 hours.

The pyrosequencing reaction

In the pyrosequencing reaction, biotinylated single-stranded DNA templates are bound to Streptavidin Sepharose™ beads in a solution containing enzymes and substrates. DNA polymerase assists in the incorporation of a dispensed deoxyribonucleotide triphosphate (dNTP) into the DNA resulting in the release of pyrophosphate (PPi). The PPi is then converted into ATP via ATP sulfurylase in the presence of adenosine 5'-phosphosulfate (APS). The ATP is used by luciferase to aid in the production of a light signal by converting luciferin to oxyluciferin. The height of each peak produced by this light signal, as can be seen in a pyrogram, is proportional to the amount of ATP processed. To avoid the recognition of deoxyadenosine triphosphate (dATP) by luciferase, an analog of ATP, deoxyadenosine alfa-thio triphosphate (dATPαS), is used by the DNA polymerase for incorporation. Apyrase is also present in the solution and is used to degrade any unincorporated nucleotides.

II. Pyrosequencing Assay Flow Chart:

(Influenza subtype determination and RNA isolation are not included in this protocol)

1) RNA Isolation

2) Influenza subtype determination

3) RT-PCR using a biotinylated primer

4) Gel analysis of the RT-PCR product

5) Sample Preparation

6) Pyrosequencing run

7) Data Analysis

III. Safety Information:

These protocols can be performed under biosafety level 1 or 2 (BSL-1/BSL-2) conditions (should be in compliance with institutional regulatory requirements) by trained personnel with appropriate personal protective equipment (PPE) using the standard aseptic technique.

IV. Abbreviations:

A(H1N1)pdm09 – the 2009 A(H1N1) pandemic influenza virus

A(H1N1) – the pre-pandemic A(H1N1) influenza virus

AQ – allele quantification

dNTP – deoxyribonucleotide triphosphate

H – histidine

NA – neuraminidase

PPi – pyrophosphate

RT-PCR – reverse transcription-polymerase chain reaction

SNP – single nucleotide polymorphism

SQA – sequence analysis

Y – tyrosine

NA amino acid numbering by NA subtype	
N1	**N2**
H275	H274

V. Reverse Transcription Polymerase Chain Reaction (RT-PCR) Protocol for the Generation of an Amplicon from Influenza Virus RNA

NOTES: This procedure is provided for Research Use Only. These protocols are not intended to be used for commercial development or for-profit testing.
Please do not distribute these procedures to other laboratories or commercial entities.

Names of vendors or manufacturers are provided as examples of suitable product sources only. Inclusion does not imply endorsement.

1 Purpose

This protocol describes the RT-PCR procedures used for cDNA synthesis and amplification of influenza virus RNA isolated from clinical specimens or grown viral isolates. The RT-PCR primers are found in "Reference Material" (section VIII). The RT-PCR amplicon generated is used in the pyrosequencing protocol(s) (section VI and/or VII).

2 Scope

This procedure is for use by the community of Public Health Laboratories with basic experience in RNA isolation, RT-PCR, and pyrosequencing.

3 Equipment

Thermal Cycler: DNA Engine® Tetrad 2 (Bio-Rad)

4 Reagents and Supplies

RT-PCR primer: Stocks of 20 µM, in aliquots of 20 µl, should be stored at -20°C.
The sequences of primers can be found in section VIII part 2.
The primers are provided for synthesis by the testing lab/group.

Superscript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity	(Invitrogen)
Protector RNase Inhibitor	(Roche)
1.5 ml sterile Eppendorf tubes	(Fisher)
Flat-Top 96-well PCR reaction plates	(ISC BioExpress)
Strip Caps for Flat-Top PCR reaction plates	(ISC BioExpress)
0.2 ml PCR tubes	(ISC BioExpress)
Assorted pipettes and pipette tips	(Rainin)
Cold block for 96-well plate	(ISC BioExpress)

2% Agarose E-Gels® 96	(Invitrogen)
E-Base® electrophoresis system	(Invitrogen)
Benchtop PCR Marker	(Promega)
Nuclease-free RT-PCR grade water	(Ambion)
Distilled deionized water	(Mediatech)

5 Procedure

5.1 RNA Isolation

RNA isolation procedures should be qualified and validated for recovery and purity before testing specimens. Commercially available isolation procedures - including QIAamp®Viral RNA Mini Kit (Qiagen), RNeasy® Mini Kit (Qiagen), MagNA Pure Compact RNA Isolation Kit (Roche), MagNA Pure LC RNA Isolation Kit II (Roche), and MagNA Pure Total Nucleic Acid Kit (Roche) - have been shown to generate highly purified RNA when following manufacturer's recommended procedures. Performance of RT-PCR based assays depend on the amount and quality of the RNA sample.

5.2 Programming the Thermal Cycler

The following program incorporates cDNA synthesis immediately followed by PCR amplification.

Step 1: Incubate, 50°C for 30 minutes

Step 2: Denature, 94°C for 2 minutes

Step 3: Denature, 94°C for 15 seconds

Step 4: Anneal, 55°C for 30 seconds

Step 5: Extend, 68°C for 1 minute

Step 6: Repeat steps 3-5 for 45 cycles

Step 7: Extend, 68°C for 5 minutes

Step 8: Hold, 4°C forever

5.3 Preparation of the RT-PCR Reaction Mix and Thermal Cycling

5.3.1 Combine the components of the Superscript™ III One-Step RT-PCR System to prepare a reaction mix (Table 1). For multiple reactions, prepare a master mix with some excess of reagent to allow for controls and pipetting error. For example, for 96 samples, prepare a master mix for 100 samples.

5.3.1.1 If using a 50 µl reaction volume, aliquot 45 µl of the master mix into the wells of a 96-well PCR plate or into 0.2 ml PCR tubes, on a cold block. Add 5 µl of RNA from test and reference viruses in the corresponding well/tube. Close wells on the plate with stripped caps or foil and close tubes with caps.

5.3.1.2 If using a 25 µl reaction volume (Table 1), combine 22.5 µl of the master mix with 2.5 µl viral RNA.

5.3.2 Briefly centrifuge to ensure that all of the components are at the bottom of the amplification tube.

5.3.3 Place the plate/tubes in the thermal cycler and run the RT-PCR program (see 5.2).

5.3.4 Analysis of the RT-PCR product should be made by gel electrophoresis to determine the production of an amplicon of the appropriate length.

Table 1. RT-PCR Reaction Components

Component	1 Reaction (50 µl volume)	1 Reaction (25 µl volume)
2X Reaction Mix	25 µl	12.5 µl
Forward Primer (20 µM)	1 µl	0.5 µl
Reverse Primer (20 µM)	1 µl	0.5 µl
SuperScript™ III High Fidelity Enzyme Mix	1 µl	0.5 µl
RNAse Inhibitor	0.5 µl	0.25 µl
Nuclease-Free Water	16.5 µl	8.25 µl
Template RNA	5 µl	2.5 µl
Total	50 µl	25 µl

Note: One of the RT-PCR primers used is biotinylated, refer to the section VIII part 2.

6 Other SOP's and Documents

User's Guide for Superscript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity.

VI. Pyrosequencing SQA Protocol Using the PyroMark™ Q24 Platform

NOTES: This procedure is provided for Research Use Only. These protocols are not intended to be used for commercial development or for-profit testing.
Please do not distribute these procedures to other laboratories or commercial entities.

When the presence of the H275Y variant is detected, it is suggested that the SNP analysis be run, which allows for allele quantification (proportion of H275 and H275Y variants, C or T at nt823 of NA gene, respectively).

Names of vendors or manufacturers are provided as examples of suitable product sources only. Inclusion does not imply endorsement.

1 Purpose

This protocol describes the pyrosequencing assay that is used for partial sequencing of the biotinylated amplicon generated in RT-PCR. This protocol is to be used in conjunction with the "Reverse Transcription Polymerase Chain Reaction (RT-PCR) Protocol for the Generation of an Amplicon from Influenza Virus RNA" (section V) and "Reference Material" (section VIII).

2 Scope

This procedure is for use by the community of Public Health Laboratories with basic experience in RNA isolation, RT-PCR, and pyrosequencing.

3 Equipment

PyroMark™ Q24 Instrument	(Qiagen)
PyroMark™ Q24 Vacuum Prep Workstation and Hand Tool	(Qiagen)
Q24 Sample Prep Thermoplate	(Qiagen)
Vacuum Pump	(Laboport)
Orbital Plate Shaker	(Labnet)
Digital Heat Block	(VWR)

4 Reagents and Supplies

Sequencing primer: Stocks of 100 µM, in aliquots of 20 µl, should be stored at -20°C.
The sequence of the primer can be found in section VIII part 2.
The primer sequence is provided for synthesis by the testing lab/group.

Streptavidin Sepharose™ High Performance beads	(GE Healthcare)
PyroMark™ Binding Buffer	(Qiagen)
PyroMark™ Annealing Buffer	(Qiagen)
PyroMark™ Denaturation Solution	(Qiagen)
PyroMark™ 10X Wash Buffer (Tris Acetate)	(Qiagen)
PyroMark™ Gold Reagents	(Qiagen)

Note: Buffers, Sepharose Beads, and PyroGold Reagents should be stored at 4°C

PyroMark Q24 Plate 24-well clear plastic plates	(Qiagen)
96% Ethanol	(Fisher)
15 ml Falcon™ conical tubes	(Fisher)
Plastic Falcon™ pipettes and Pipette-Aid™	(Fisher)
Assorted pipettes and pipette tips	(Rainin)
24-well plastic PCR reaction plates	(Fisher)
Distilled deionized water	(Mediatech)
Nuclease-free water	(Ambion)
Plastic troughs/trays for sample clean up	(Qiagen)
Filter probes for Vacuum prep tool	(Qiagen)
Plastic bottles for preparing buffers and reagents	(Nalgene)

5 Reference Viruses and Biotinylated Amplicon (Template)

5.1 These reference viruses are to be used directly for viral RNA isolation and pyrosequencing. This is because virus passage in cell culture can alter the genetic make-up of the virus, which may affect the pyrosequencing results. For example, an H275Y variant could revert to a wildtype sequence (H275). Examples of reference viruses for this target may be found in part 1 of section VIII.

5.2 A biotinylated amplicon is generated using isolated viral RNA in an RT-PCR reaction with its corresponding primers as described in section V and section VIII.

6 Procedure

6.1 Preparation of Run Sample Sheet and Import into PyroMark™ ID Platform

6.1.1 Prepare an MS Excel spreadsheet of samples to be tested (Figure 1) and save as a text, tab delimited file (*.txt).

6.2 Setup of Pyrosequencing Reaction

6.2.1 Set the digital heat block to 89°C, place the Q24 Sample Prep Thermoplate holder upon the heat block and allow the plate to heat to 89°C.

6.2.2 Remove the Streptavidin Sepharose™ beads, PyroMark™ Binding Buffer, Annealing Buffer, Denaturation Solution, 1X Wash Buffer and 70% Ethanol from 4°C storage and place at room temperature (**Note**: Dilute PyroMark™ 10X Wash Buffer in distilled deionized water 1/10 to make the 1X working solution of wash buffer listed above. Dilute 96% ethanol to 70% ethanol by combining 700 ml of 96% ethanol and 280 ml of distilled deionized water).

6.2.3 When at room temperature, prepare binding buffer solution (60 µl of binding buffer solution is needed per sample). For 24 samples, combine 2 ml Binding Buffer, 1 ml distilled deionized water and 150 µl Streptavidin Sepharose™ beads (resuspend beads by inverting) in a 15 ml conical tube. Briefly mix the binding buffer solution by inverting.

6.2.4 Aliquot 60 µl of the binding buffer solution into the wells of a 24-well PCR plate that will be used for pyrosequencing the RT-PCR product.

6.2.5 Transfer 20µl of RT-PCR product into the respective wells of the 24-well PCR plate containing binding buffer solution. Cover the plate with a plastic adhesive cover, place on an orbital plate shaker and agitate at 1400 rpm for at least 10 minutes. Allow the plate to continue shaking until ready to begin the sample clean-up steps with the PyroMark™ Vacuum Prep Workstation (Step 6.2.11).

6.2.6 Prepare annealing buffer solution (40 µl of annealing buffer solution is needed per sample). For 24 samples, combine 1.1 ml Annealing Buffer and 5 µl of the required 100 µM sequencing primer (final concentration 0.45 µM) in a 15 ml conical tube. Briefly vortex to mix the annealing buffer solution.

6.2.7 Aliquot 40 µl of the annealing buffer solution into the respective wells of a PyroMark Q24 plate 24-well clear plastic plate. Place the plate onto its respective position on the Vacuum Prep Workstation (Figure 2).

6.2.8 Set plastic reagent trays in their designated positions on the Vacuum Prep Workstation (Figure 2) and fill with 70% Ethanol, Denaturation Solution, 1X Wash Buffer and distilled deionized water, respectively.

6.2.9 When Step 6.2.5 is close to completion (3-5 minutes left), prime the filters in the PyroMark™ Prep Tool by adding distilled deionized water into a plastic reagent tray and place beneath the tool (Figure 2). Turn ON the vacuum switch for both the Vacuum Prep Workstation and the vacuum pump. This will allow water to flush through and prime the filter probes on the Prep Tool. Let the tool sit in the plastic reagent tray until it is ready to use.

6.2.10 When Step 6.2.5 is complete, remove the plastic PCR plate containing the PCR product bound to Streptavidin Sepharose™ beads from the shaker. Place the plate onto its respective position on the Vacuum Prep Workstation. Gently remove plastic adhesive cover from plate to avoid splashing.

6.2.11 With the vacuum ON, as described in Step 6.2.9, place the Prep Tool into the respective wells of the 24-well PCR plate until the entire volume has been collected (5-10 seconds). The Streptavidin Sepharose™ beads with immobilized amplicons will be captured on the tips of the respective filter probes of the Prep Tool and will be visible.

Note: The tips of the probes are white like the beads. The captured beads will appear as a concave white substance on the tips of the probes.

6.2.12 With the vacuum still ON, transfer the Prep Tool into the reagent tray containing 70% ethanol and flush until the tray is almost emptied (15-25 seconds), then lift to drain the tool. This step allows any unbound amplicon or unincorporated reagents from the RT-PCR reaction to be washed off.

6.2.13 With the vacuum still ON, transfer the Prep Tool into the reagent tray containing Denaturation Solution and flush until the tray is almost emptied (15-25 seconds), then lift to drain the tool. This step allows for the denaturation of the double-stranded DNA amplicon containing a biotin-tagged, single-stranded, DNA template. The complementary strand synthesized from the non-biotinylated primer will be washed away.

6.2.14 With the vacuum still ON, transfer the Prep Tool into the reagent tray with 1X Wash Buffer and flush until the tray is almost emptied (15-25 seconds), then lift to drain the tool for 30-40 seconds.

6.2.15 Turn the vacuum OFF and **disconnect** the Prep Tool from the vacuum line. Place the Prep Tool into the 25-well clear plastic plate containing annealing buffer solution (including sequencing primer). Move the Prep Tool in circular motions (15-25 seconds) in the 24-well clear plastic plate to release beads bound to the single-stranded template.

Note: The mixture on the plate should now appear 'cloudy' and the white concave substance should no longer be present on the tips of the probes.

6.2.16 Denaturation of template/primer mixture: Place the 24-well clear plastic plate onto the Q24 Sample Prep Thermoplate holder on the 89°C heat block for 4 minutes. Do not leave the 24-well clear plastic plate on the heat block for more than 4 minutes.

6.2.17 Annealing template and primer: Remove the Q24 Sample Prep Thermoplate holder with the 24-well clear plastic plate from the 89°C heat block and allow it to cool on a bench for 10 minutes.

6.2.18 Remove the 24-well clear plastic plate from the Q24 Sample Prep Thermoplate holder and allow it to rest directly on the bench for an additional 4 minutes to end the annealing step.

Note: During the 10 minutes, when the plate on the holder is cooling (Step 6.2.17), Steps 6.3.1 through 6.3.11 can be completed.

6.3 Pyrosequencing Using the PyroMark™ Q24 Equipment and Software

6.3.1 Open PyroMark™ Q24 software.

6.3.2 Import the saved text file into the PyroMark™ Q24 platform as follows:

1. Click on "New Run"
2. Right Click on the first well.

3. Click on "Insert Sample Layout File"
4. Browse to find the run sample sheet (saved as a "*.txt" in Step 6.1.1)
5. Click "Open"

6.3.3 In the drop down "Instrument Method" menu, select "PyroMark Q24 Method 000X". The specific number for "X" should match what is on the reagent cartridge to be used. Instructions for the setup of a new instrument parameter are provided with the reagent cartridge.

6.3.4 Select a dispensation entry:

6.3.4.1 Go to the "Shortcuts" folder, in the "SQA Dispensations" folder click to select the dispensation order (Figure 3).

Note: "SQA Dispensations" folder needs to be created prior to this step.

6.3.4.2 If the target specific entry is not in the "SQA Dispensation" folder found under the "Shortcuts" folder, then create a new SQA. To create a new SQA entry, right click on the "SQA Dispensations" folder, go to "New Assay" and click on "SQA Assay". Enter the name found in part 4 of section VIII. Under the "Dispensation Order" box, enter in the sequence found in part 5 of section VIII. The sequence should be visible under the "Expanded dispensation order" box (Figure 4).

6.3.5 In the "New Run Setup", highlight all the wells that are to be used and drag the dispensation order to the wells. The name of the entry should now appear in these wells.

6.3.6 Click the "Tools" tab to reveal a drop-down menu. Select "Pre Run Information" to see the volumes of the enzyme (E), substrate (S) and nucleotides (A, C, G and T) that need to be added to the cartridge.

6.3.7 Save the run onto a USB drive.

6.3.8 Remove the PyroGold reagent kit from 4^{o}C storage.

6.3.9 Reconstitute both the enzyme (E) and the substrate (S) in 620 µl nuclease-free water. Swirl to mix, DO NOT vortex. The reconstituted enzyme (E) and the substrate (S) are stable for at least 5 days at 4°C, or for one freeze (-20°C) /thaw cycle.

6.3.10 Ensure that the dispensation pins (needles) on the cartridge are clean and not bent before use. To do so, fill each channel in the cartridge with distilled deionized water, seal the channel and apply pressure. Water should stream straight down out of the pin. Remove the excess water and add the corresponding amount of enzyme, substrate and nucleotides (see 6.3.6) into each channel of the cartridge with the label facing the user, as shown in Figure 5. Load the volumes carefully to ensure that air bubbles are not created.

Note: If the water does not stream through the pins of the cartridge, the pins may be clogged. Soaking the pins in warm water may help unclog the pins. A cartridge with clogged pins should not be used and a new cartridge may be needed.

6.3.11 Load the cartridge containing enzyme, substrate and nucleotides onto the PyroMark™ Q24 instrument, with the cartridge label facing towards the user.

6.3.12 Load the 24-well clear plastic plate containing the annealed DNA template and sequencing primer onto the PyroMark™ Q24 instrument.

6.3.13 Insert the USB stick containing the run into the USB port at the front of the PyroMark Q24 Instrument.

6.3.14 Find and select the run to start the pyrosequencing reaction. Ensure that the substrate peak appears after the substrate is added to confirm that the enzyme and substrate reagents are working.

6.4 Cleaning the Instrument

6.4.1 To clean the Prep Tool, place the hand tool in the reagent tray containing distilled deionized water, reconnect the vacuum line to the hand tool and turn ON the vacuum. Flush until the tray is almost emptied (1-2 minutes).

6.4.2 Turn the vacuum OFF. Remove the reagent trays from the Vacuum Prep Workstation, discard excess solutions and rinse the reagent trays with distilled deionized water. Set the reagent trays upon paper towels on the bench to dry.

6.4.3 When the Pyrosequencing run is finished, remove and discard the 24-well clear plastic plate from the PyroMark™ Q24 instrument.

6.4.4 Remove the cartridge from the PyroMark™ Q24 instrument and discard unused enzyme, substrate and nucleotides. Wash the cartridge at least 3 times with distilled deionized water and check to make sure that all dispensation pins are clean by applying pressure to the top of each of the channels in the cartridge (see **Note** for 6.3.10).

6.5 Analysis of Pyrosequencing Data

6.5.1 When the SQA run is complete, remove the USB stick and copy the finished run onto your computer.

Note: The icon for a completed run file is a blue check, and will replace the green icon, which denotes a run setup that has not yet been run.

6.5.2 Reopen the run in the PyroMark™ Q24 software to reveal the SQA analysis window (Figure 6).

6.5.3 Under the “Analyze” option, select “Analyze All Wells” to analyze all the samples in the run. To analyze only the wells of interest, first highlight the wells you wish to analyze and then click “Analyze Selected Wells”.

6.5.4 When analysis is complete, click “Save”.

6.5.5 To report a run, under the “Report” tab, select the “SQA Full Report”. This will generate a document containing pyrograms and the results of the analysis.

Note: It is CRITICAL that each pyrogram be examined and verified visually (see examples of pyrograms in 6.7).

6.6 Data Analysis Using IdentiFire™ Software

6.6.1 In the PyroMark™ Q24 software under the “Tools” tab, click “Export As FASTA” and save the file.

6.6.2 Prior to IdentiFire™ analysis, copy the IdentiFire™ Library (found in part 6 of section VIII) containing the pyrosequencing target region of interest (wildtype and variant genotypes) and paste into a new MS Word file, save as a text (*.txt).

6.6.3 Open IdentiFire™ software. Under the “Sequence Input” select the “Sequence File” tab. Find and select the run to be analyzed. To analyze all samples in the run, click “Add all”. To analyze only selected samples, highlight samples of interest and click “Add”.

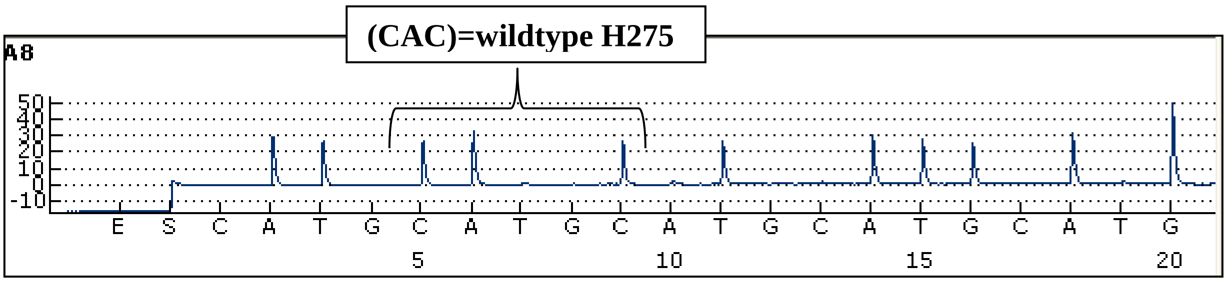
6.6.4 Under “Analysis Setup”, select the corresponding library (created in Step 6.6.2) in the “Sequence Library” drop-down, or select “Browse…” to locate where the library is saved. Click on “Add to All”. Select the play/start button to begin analysis.

6.6.5 After analysis, generate reports and save. When saving reports, select the option "All Reports".

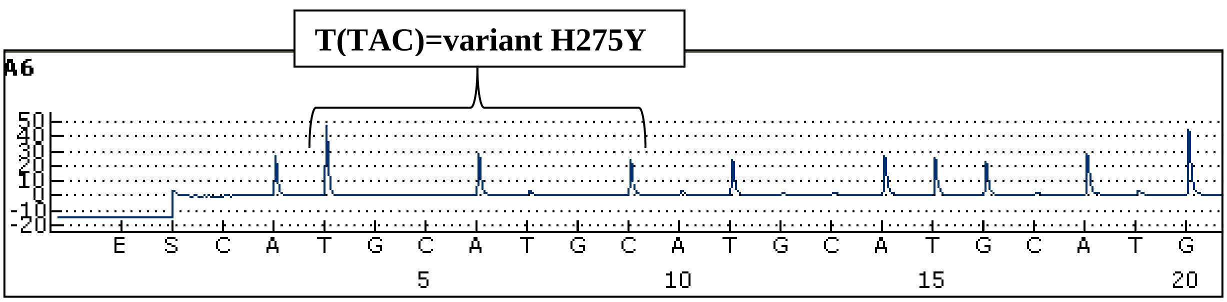
6.7 Analysis of Pyrosequencing Data

Below are examples showing the common target sequences.

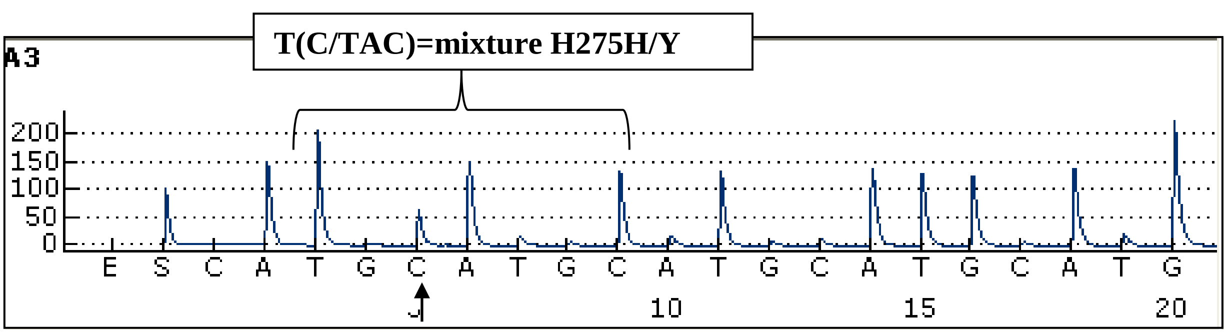
1) Detection of A(H1N1)pdm09-wildtype (H275): AT <u>CAC</u> TAT



2) Detection of A(H1N1)pdm09-variant (H275Y): AT <u>TAC</u> TAT



3) Detection of A(H1N1)pdm09-mixture-(H275H/Y): AT (<u>C/T)AC</u> TAT



Note: Arrow shows the presence of C incorporation for T(<u>C</u>AC) virus variant.

7 Notes

7.1 This pyrosequencing protocol is for the detection of substitutions found in influenza and is not to be used for typing/subtyping; rather, it is to be used for viruses that have already been subtyped by another method (e.g., real-time RT-PCR).

7.2 A positive control is optional for the pyrosequencing assay, since the PyroMark™ Q24 provides a built-in quality control for each assay. Specifically, if a pyrogram is generated for any of your samples, this may serve as your positive control.

7.3 The naming of the sequences in the IdentiFire™ library can be changed to the users' preference.

7.4 When the IdentiFire™ library is used, any mutations within the target region that were not in the sequences of the reference library will be identified by the software as mismatched sequences and the closest matches will be shown.

7.5 Because the PyroMark™ Q24 platform has limitations, it is suggested that each pyrogram be examined and verified visually. One such limitation is that if the target sequence contains repeats of more than four or five identical nucleotides, the pyrogram peak heights may no longer be directly proportional to the number of nucleotides. In such cases, the software may overcall or miscall the nucleotide in the final sequence results.

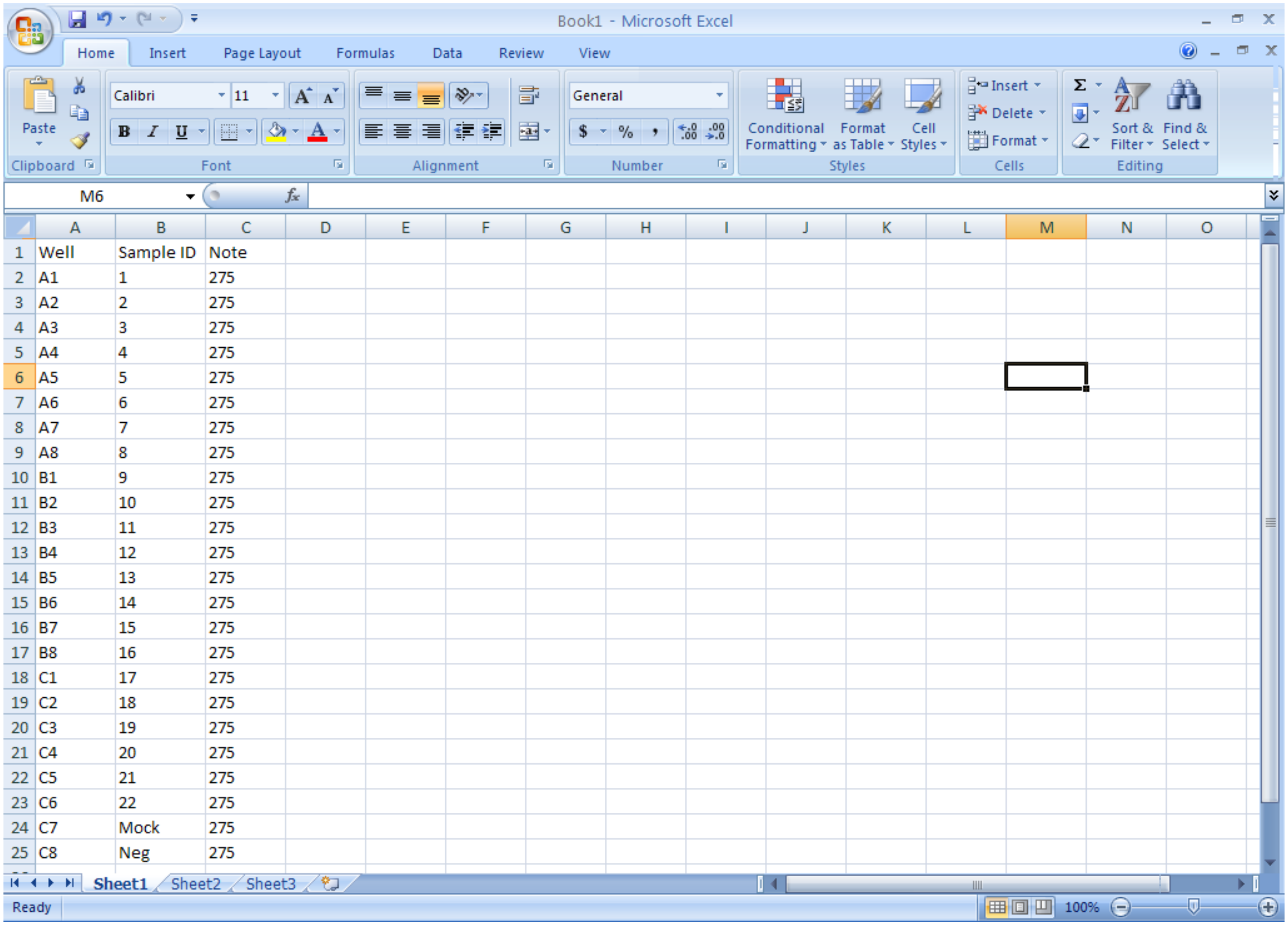
7.6 Both the PyroMark™ Q24 and the IdentiFire™ software will not be able to detect mixtures (e.g., H/Y at 275). Mixtures must be verified visually.

7.7 As the virus evolves, the IdentiFire™ library sequences and primers may need to be updated.

8 Other SOPs and Documents

PyroMark™ Q24 User's Manual.

Index of Figures



Well	Sample ID	Note
A1	1	275
A2	2	275
A3	3	275
A4	4	275
A5	5	275
A6	6	275
A7	7	275
A8	8	275
B1	9	275
B2	10	275
B3	11	275
B4	12	275
B5	13	275
B6	14	275
B7	15	275
B8	16	275
C1	17	275
C2	18	275
C3	19	275
C4	20	275
C5	21	275
C6	22	275
C7	Mock	275
C8	Neg	275

Figure 1. Example of a Run Sample Sheet for PyroMark™ Q24 Platform.

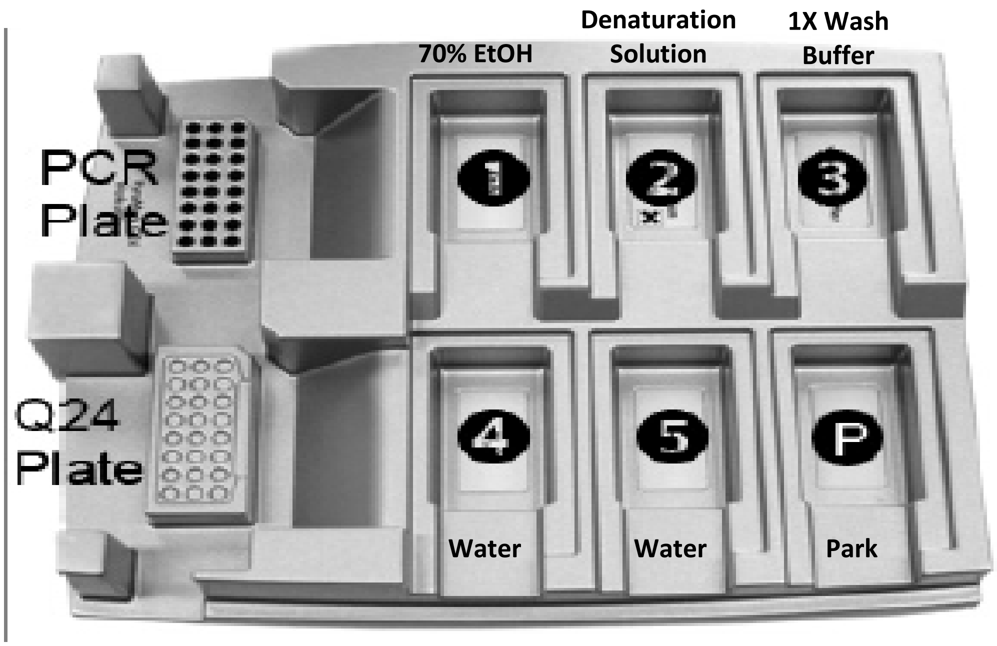


Figure 2. The PyroMark™ Q24 Vacuum Prep Workstation.

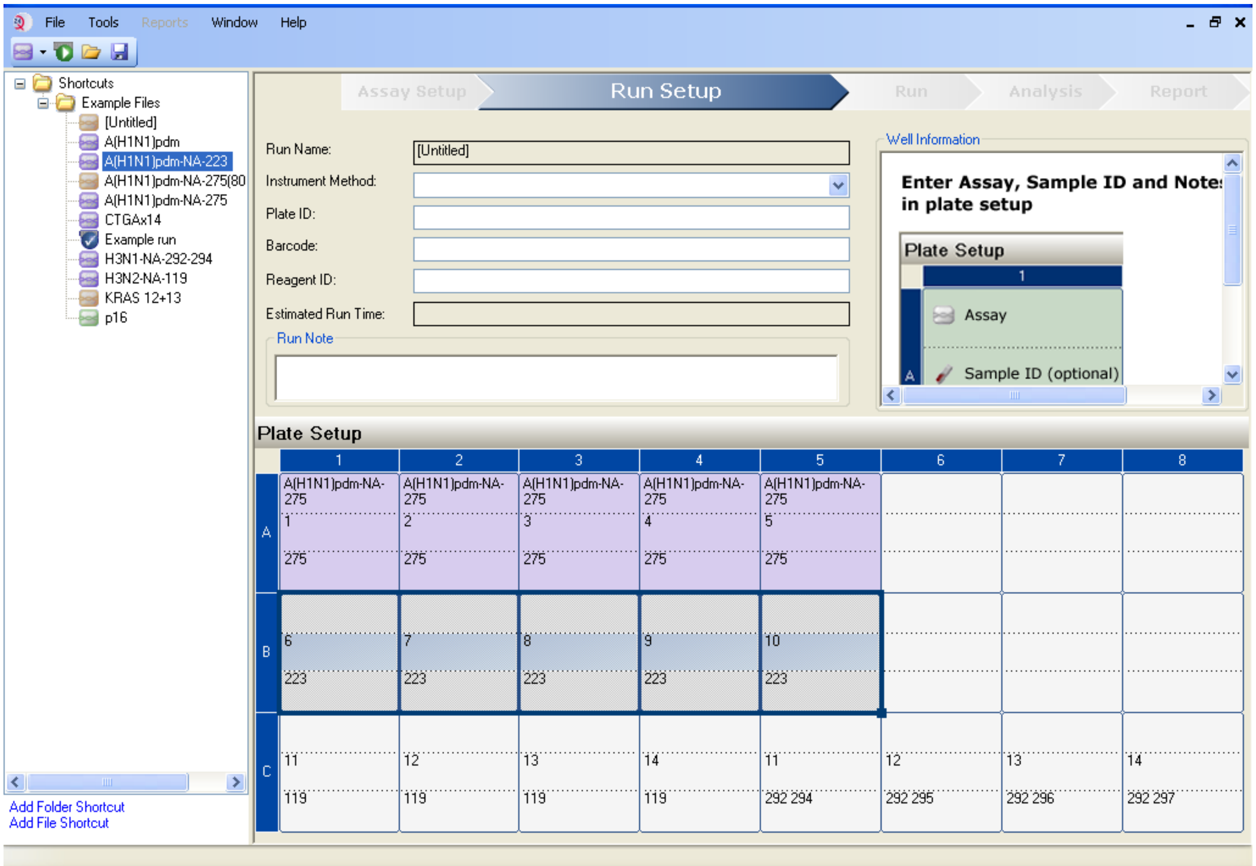


Figure 3. Example of PyroMark™ Q24 Software Showing an SQA Run Setup.

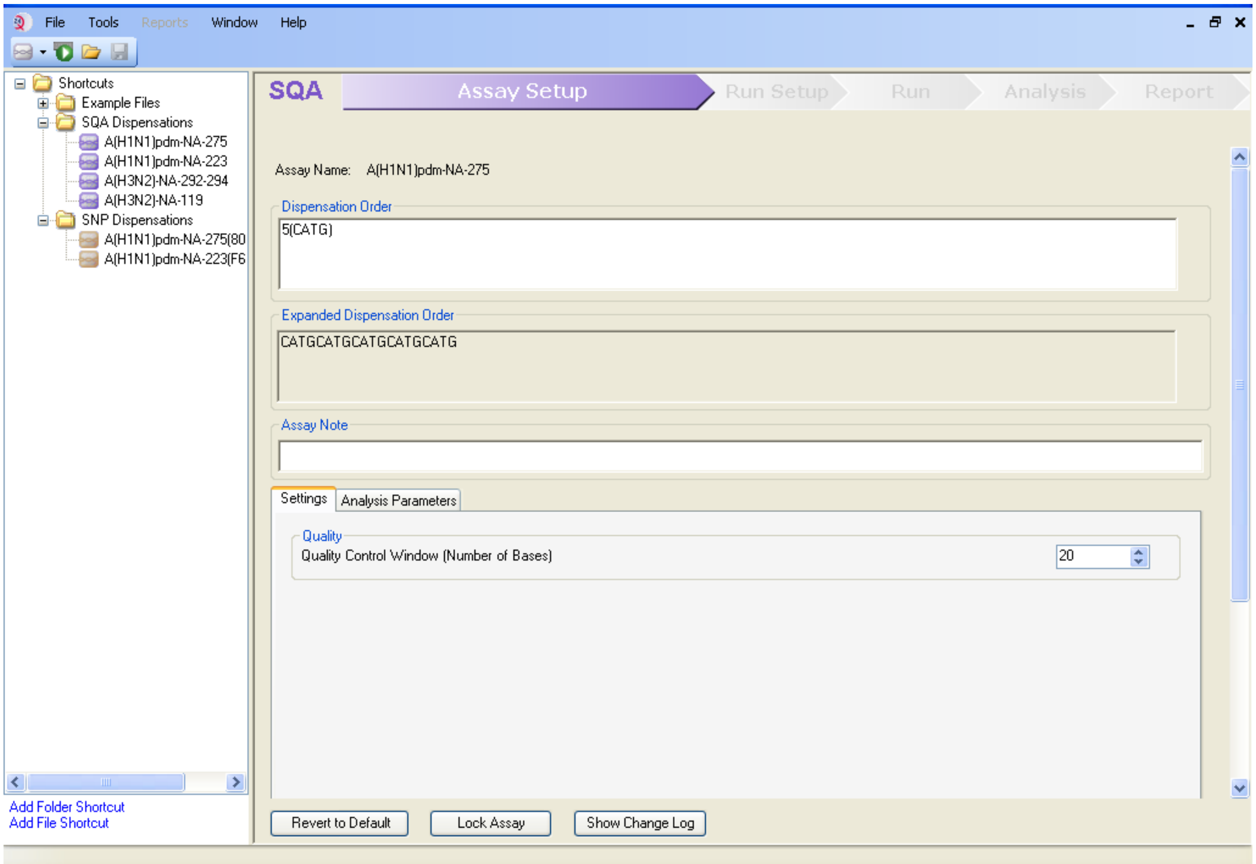


Figure 4. Example of Defining an SQA Entry.

E	**A**	**S**
T		**C**
	G	

Label Should Face User

Figure 5. PyroMark™ Q24 Cartridge Setup.

Figure 6. Example of an SQA Run Analysis.

VII. Pyrosequencing for Single Nucleotide Polymorphism (SNP) Analysis Protocol Using the PyroMark™ Q24 Platform

NOTES: This procedure is provided for Research Use Only. These protocols are not intended to be used for commercial development or for-profit testing.
Please do not distribute these procedures to other laboratories or commercial entities.

Analysis of samples using the SNP assay requires knowledge of the sequence. To assure that there is no natural variation within the targeted sequence, it is recommended that the SQA assay be run first. Moreover, compared to SQA, SNP analysis is more susceptible to interference by various factors (e.g., quality of RT-PCR product, background, cartridge issues, etc.).

Names of vendors or manufacturers are provided as examples of suitable product sources only. Inclusion does not imply endorsement.

1 Purpose

This protocol is to be used in conjunction with the "Reverse Transcription Polymerase Chain Reaction (RT-PCR) Protocol for the Generation of an Amplicon from Influenza Virus RNA" (section V), "Pyrosequencing SQA Protocol Using the PyroMark™ Q24 ID Platform" (section VI) and "Reference Material" (section VIII). This protocol describes the pyrosequencing assay that is used for partial sequencing of the biotinylated amplicon generated in RT-PCR to detect and quantify the ratio of wildtype and mutant variants in a viral population.

The single nucleotide polymorphism (SNP) and allele quantification (AQ) analyses are done at nucleotide 823 (the first nucleotide of codon CAC) of the neuraminidase (NA) gene of the A(H1N1)pdm viruses. Cytosine (C) at the first position is present in the wildtype viruses carrying histidine at position 275 in the NA protein, whereas thymine (T) at this position is present in variant H1N1 viruses carrying tyrosine at position 275 in the NA protein.

2 Scope

This procedure is for use by the community of Public Health Laboratories with basic experience in RNA isolation, RT-PCR, and pyrosequencing.

3 Equipment

PyroMark™ Q24 Instrument	(Qiagen)
PyroMark™ Q24 Vacuum Prep Workstation and Hand Tool	(Qiagen)
Q24 Sample Prep Thermoplate	(Qiagen)
Vacuum Pump	(Laboport)
Orbital Plate Shaker	(Labnet)
Digital Heat Block	(VWR)

4 Reagents and Supplies

Sequencing primer: Stocks of 100 µM, in aliquots of 20 µl, should be stored at -20°C.

The sequence of the primer can be found in section VIII part 2.
The primer sequence is provided for synthesis by the testing lab/group.

Streptavidin Sepharose™ High Performance beads	(GE Healthcare)
PyroMark™ Binding Buffer	(Qiagen)
PyroMark™ Annealing Buffer	(Qiagen)
PyroMark™ Denaturation Solution	(Qiagen)
PyroMark™ 10X Wash Buffer (Tris Acetate)	(Qiagen)
Pyro Gold Reagents	(Qiagen)

Note: Buffers, Sepharose Beads, and PyroGold Reagents should be stored at 4°C

PyroMark™ Q24 Plate 24-well clear plastic plates	(Qiagen)
96% Ethanol	(Fisher)
15 ml Falcon™ conical tubes	(Fisher)
Plastic Falcon™ pipettes and Pipette-Aid™	(Fisher)
Assorted pipettes and pipette tips	(Rainin)
24-well plastic PCR reaction plates	(Fisher)
Distilled deionized water	(Mediatech)
Nuclease-free water	(Ambion)
Plastic troughs/trays for sample clean up	(Qiagen)
Filter probes for Vacuum prep tool	(Qiagen)
Plastic bottles for preparing buffers and reagents	(Nalgene)

5 Reference Viruses and Biotinylated Amplicon (Template)

5.1 These reference viruses are to be used directly for viral RNA isolation and pyrosequencing. This is because virus passage in cell culture can alter the genetic make-up of the virus, which may affect the pyrosequencing results. For example, an H275Y variant could revert to a wild-type sequence (H275). Examples of reference viruses for a particular target may be found in part 1 of section VIII.

5.2 A biotinylated amplicon is generated using isolated viral RNA in an RT-PCR reaction as described in section V.

6 Procedure

6.1 Preparation of Run Sample Sheet and Import into PyroMark™ ID Platform

6.1.1 Prepare an MS Excel spreadsheet of samples to be tested (Figure 1) and save as a text, tab delimited file (*.txt).

6.2 Setup of Pyrosequencing Reaction

See part 6.2 in section VI, "Pyrosequencing SQA Protocol Using the PyroMark™ Q24 Platform"

6.3 Pyrosequencing Using the PyroMark™ Q24 Equipment and Software

6.3.1 Open PyroMark™ Q24 software.

6.3.2 Import the saved text file into the PyroMark™ Q24 platform as follows:

6. Click on "New Run"
7. Right Click on the first well.
8. Click on "Insert Sample Layout File"
9. Browse to find the run sample sheet (saved as a "*.txt" in Step 6.1.1)
10. Click "Open"

6.3.3 In the drop down "Instrument Method" menu, select "PyroMark Q24 Method 000X". The specific number for "X" should match what is on the reagent cartridge to be used. Instructions for the setup of a new instrument parameter are provided with the reagent cartridge.

6.3.4 Select a dispensation entry:

6.3.4.1 Go to the "Shortcuts" tab, and under the "SNP Dispensations" folder click to select the dispensation order (Figure 3).

6.3.4.2 If the target specific entry is not in the "SNP Dispensation" folder under the "Shortcuts" folder, then create a new SNP entry. To create a new SNP (referred to as "AQ" by the software) entry, right click on the "SNP Dispensations" folder, go to "New Assay" and click on "AQ Assay". Enter the name "A(H1N1)pdm09-NA275". Under the "Dispensation Order" box, enter the sequence to be analyzed- "ATC/TACTAT". The generated dispensation should be visible under the "Expanded Dispensation Order" box (Figure 4).

Note: Because of the natural genetic variability of the influenza virus genome, SNP analysis can be affected if mutations occur in the region near the site of analysis. Such mutations are rare, but will cause the sample to fail; for example, a virus variant with the silent mutation at nucleotide position 822 (T→C) of the NA gene. If a sample with this mutation is determined by SQA, the SNP analysis should be done with the following variant sequence: A<u>C</u>C/TACTAT (Figure 5B). The result will be inconclusive if such a variant is tested in SNP with the dispensation order generated for the major circulating virus variant (A<u>T</u>C/TACTAT) (Figure 5A).

6.3.5 On the New Run Setup, highlight all the wells that are to be used and drag the dispensation order to the wells. The name of the entry should now appear in these wells.

6.3.6 Click the "Tools" tab to reveal a drop-down menu. Select "Pre Run Information" to see the volumes of the enzyme (E), substrate (S) and nucleotides (A, C, G and T) that need to be added to the cartridge.

6.3.7 Copy the run on a USB stick.

6.3.8 Remove the PyroGold reagent kit from 4^{o}C storage.

6.3.9 Reconstitute both the enzyme (E) and the substrate (S) in 620 µl nuclease-free water. Swirl to mix, DO NOT vortex. The reconstituted enzyme (E) and the substrate (S) are stable for at least 5 days at 4°C, or for one freeze (-20°C)/thaw cycle.

6.3.10 Ensure that the dispensation pins (needles) on the cartridge are clean and not bent before use. To do so, fill each channel in the cartridge with distilled deionized water, seal the channel and apply pressure. Water should stream straight down out of the pin. Remove the excess water and add the corresponding amount of enzyme, substrate and nucleotides (see 6.3.6) into each channel of the cartridge with the label facing the user, as shown in Figure 6. Load the volumes carefully to ensure that air bubbles are not created.

Note: If the water does not stream through the pins of the cartridge, the pins may be clogged. Soaking the pins in warm water may help unclog the pins. A cartridge with clogged pins should not be used and a new cartridge may be needed.

6.3.11 Load the cartridge containing enzyme, substrate and nucleotides onto the PyroMark™ Q24 instrument, with the cartridge label facing towards the user.

6.3.12 Load the 24-well clear plastic plate containing the annealed DNA template and sequencing primer onto the PyroMark™ Q24 instrument.

6.3.13 Insert the USB stick containing the run into the USB port at the front of the PyroMark™ Q24 instrument.

6.3.14 Find and click on the run to start the pyrosequencing reaction. Ensure that the substrate peak appears after the substrate is added to confirm that the enzyme and substrate reagents are working.

6.4 Cleaning the Instrument

See part 6.4 in section VI, "Pyrosequencing SQA Protocol Using the PyroMark™ Q24 ID Platform"

6.5 Analysis of Pyrosequencing Data Using Allele Quantification (AQ) Mode

6.5.1 When the SNP run is complete, remove the USB stick and copy the finished run on to your computer.

Note: The icon for a completed run file is a blue check, and will replace the green icon, which denotes a run setup that has not yet been run.

6.5.2 Reopen the run in the PyroMark™ Q24 software to reveal the AQ analysis window (Figure 7).

6.5.3 Under the "Analyze" tab, select "Analyze All Wells" to analyze all the samples in the run. To analyze only selected wells, first highlight the wells you wish to analyze, and then click "Analyze Selected Wells".

6.5.4 When analysis is complete, click "Save".

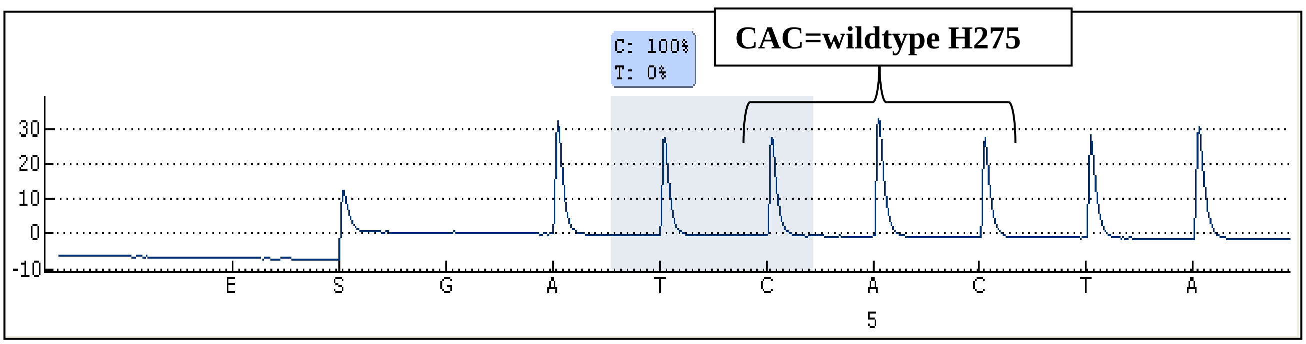
6.5.5 Pyrosequencing data may be reported in two modes: SNP or AQ. SNP mode will provide the nucleotide present at the site of analysis (for example: T/T, T/C or C/C; Figure 8). AQ mode will provide the percentage of each nucleotide present (Figure 9).

6.5.6 Under the "Report" tab, select the "SNP Full Report" or "AQ Full Report". This will generate a document containing pyrograms and the results of the analysis.

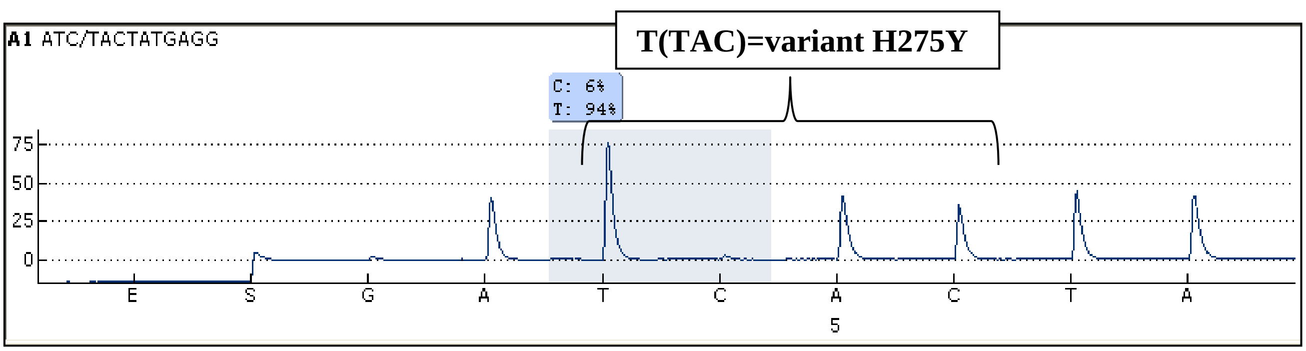
6.6 Analysis of Pyrosequencing Data

Below are examples showing the common target sequences.

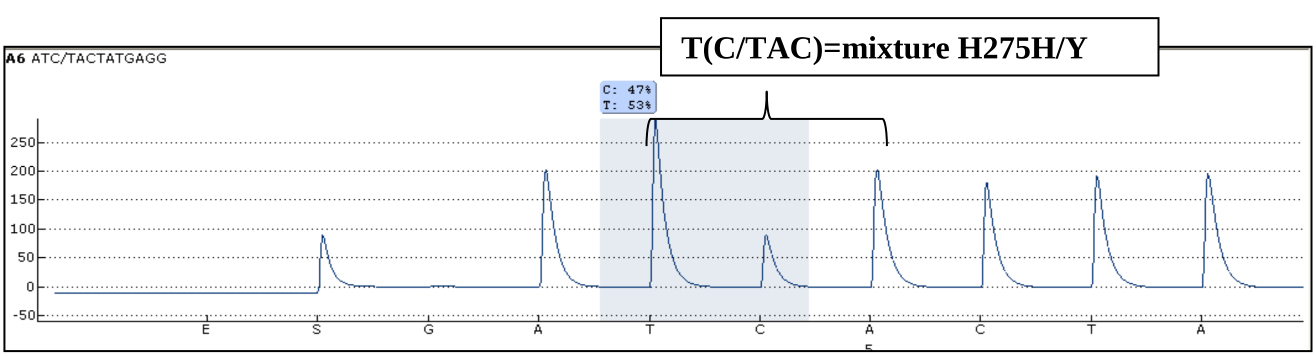
1) Detection of A(H1N1)pdm09-wildtype (H275): AT CAC TAT



2) Detection of A(H1N1)pdm09-variant (H275Y): AT TAC TAT



3) Detection of A(H1N1)pdm09-mixture-(H275H/Y): AT (C/T)AC TAT



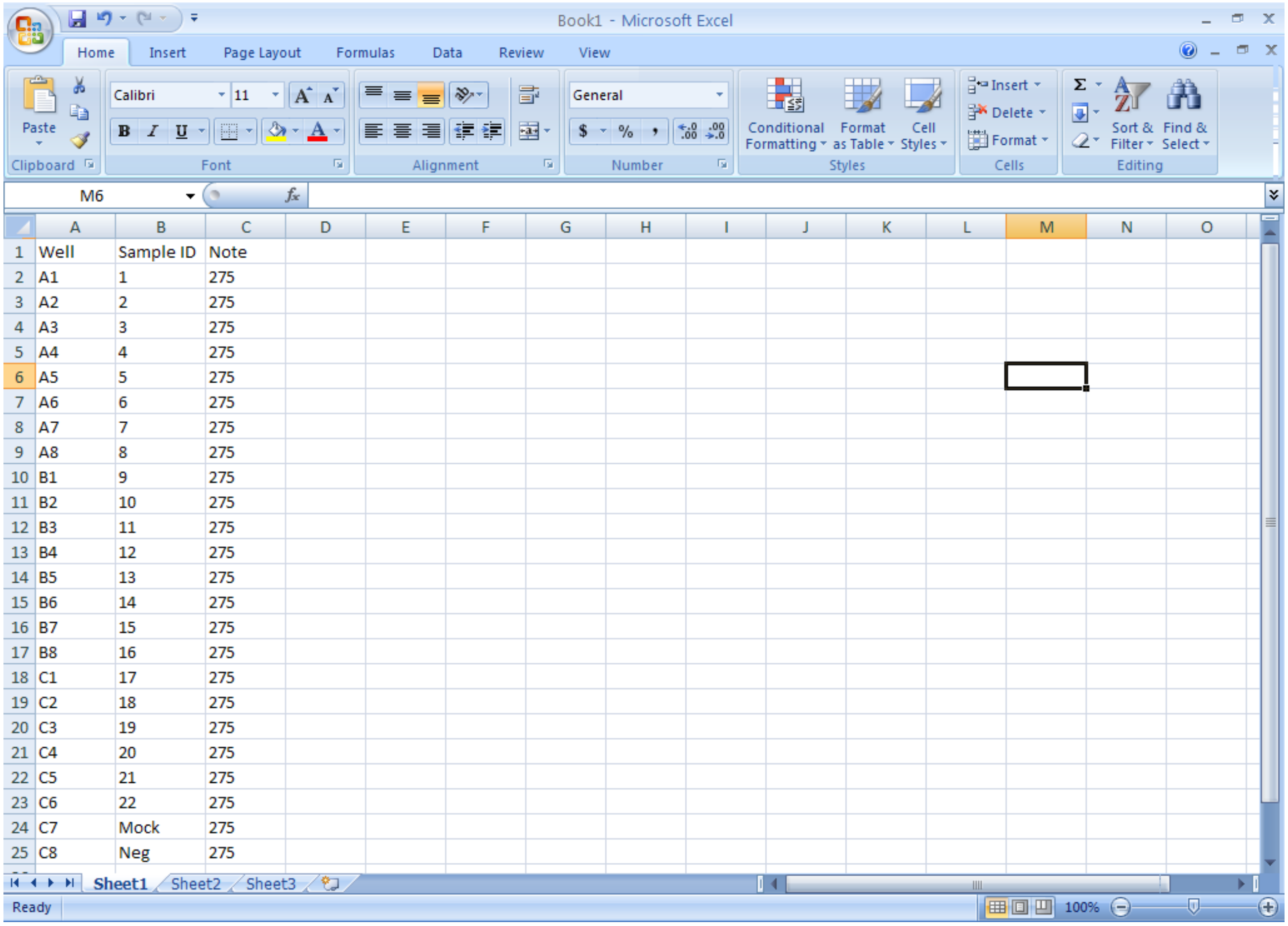
7 Notes

See notes in part 7 in section VI, “Pyrosequencing SQA Protocol Using the PyroMark™ Q24 ID Platform”

8 Other SOPs and Documents

PyroMark™ Q24 User’s Manual.

Index of Figures



Well	Sample ID	Note
A1	1	275
A2	2	275
A3	3	275
A4	4	275
A5	5	275
A6	6	275
A7	7	275
A8	8	275
B1	9	275
B2	10	275
B3	11	275
B4	12	275
B5	13	275
B6	14	275
B7	15	275
B8	16	275
C1	17	275
C2	18	275
C3	19	275
C4	20	275
C5	21	275
C6	22	275
C7	Mock	275
C8	Neg	275

Figure 1. Example of a Run Sample Sheet for PyroMark™ Q24 Platform.

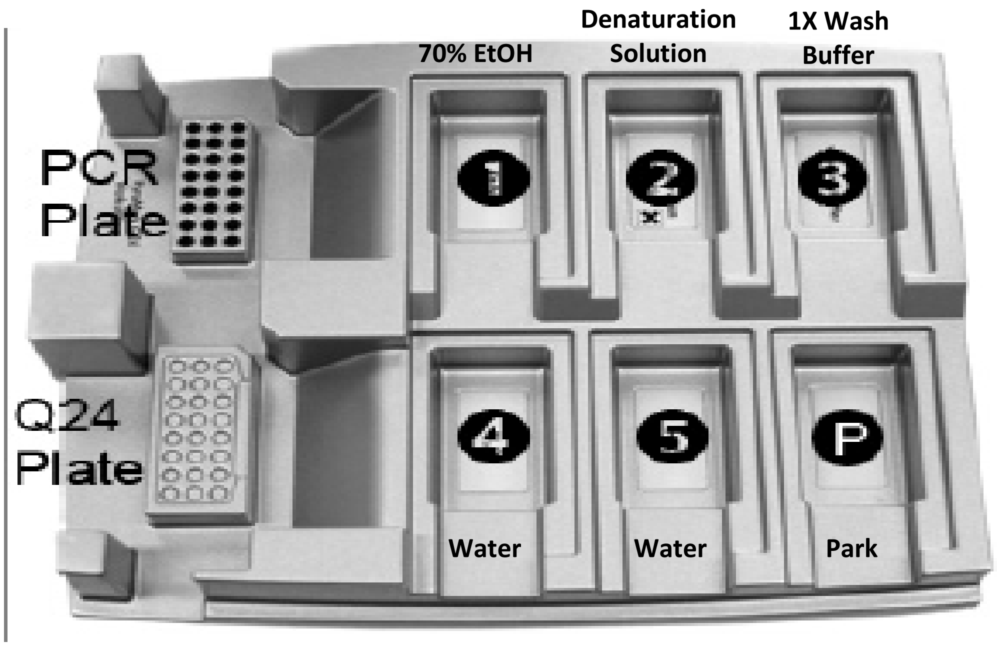


Figure 2. The PyroMark™ Q24 Vacuum Prep Workstation.

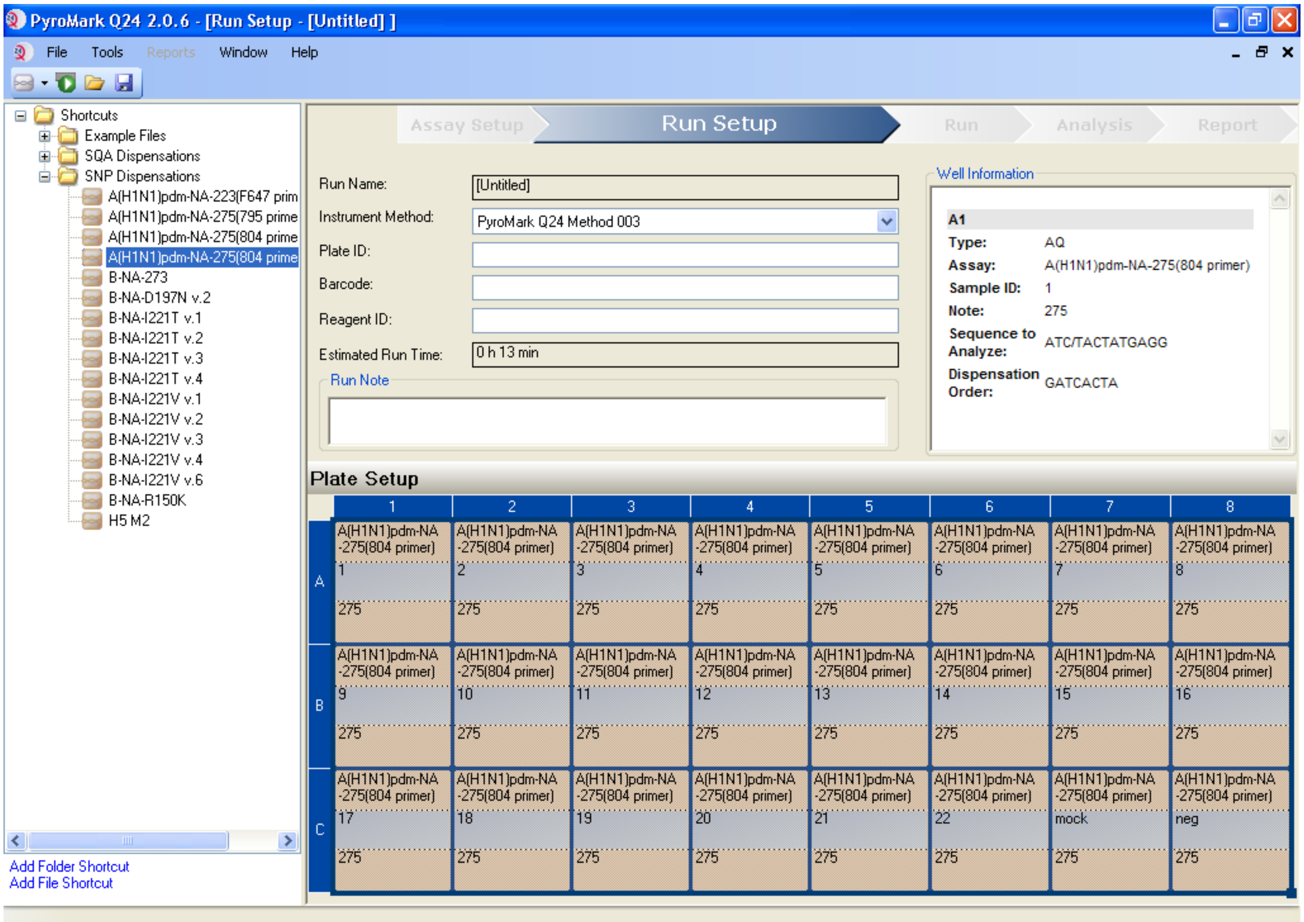


Figure 3. Example of PyroMark™ Q24 Software Showing a SNP Run Setup.

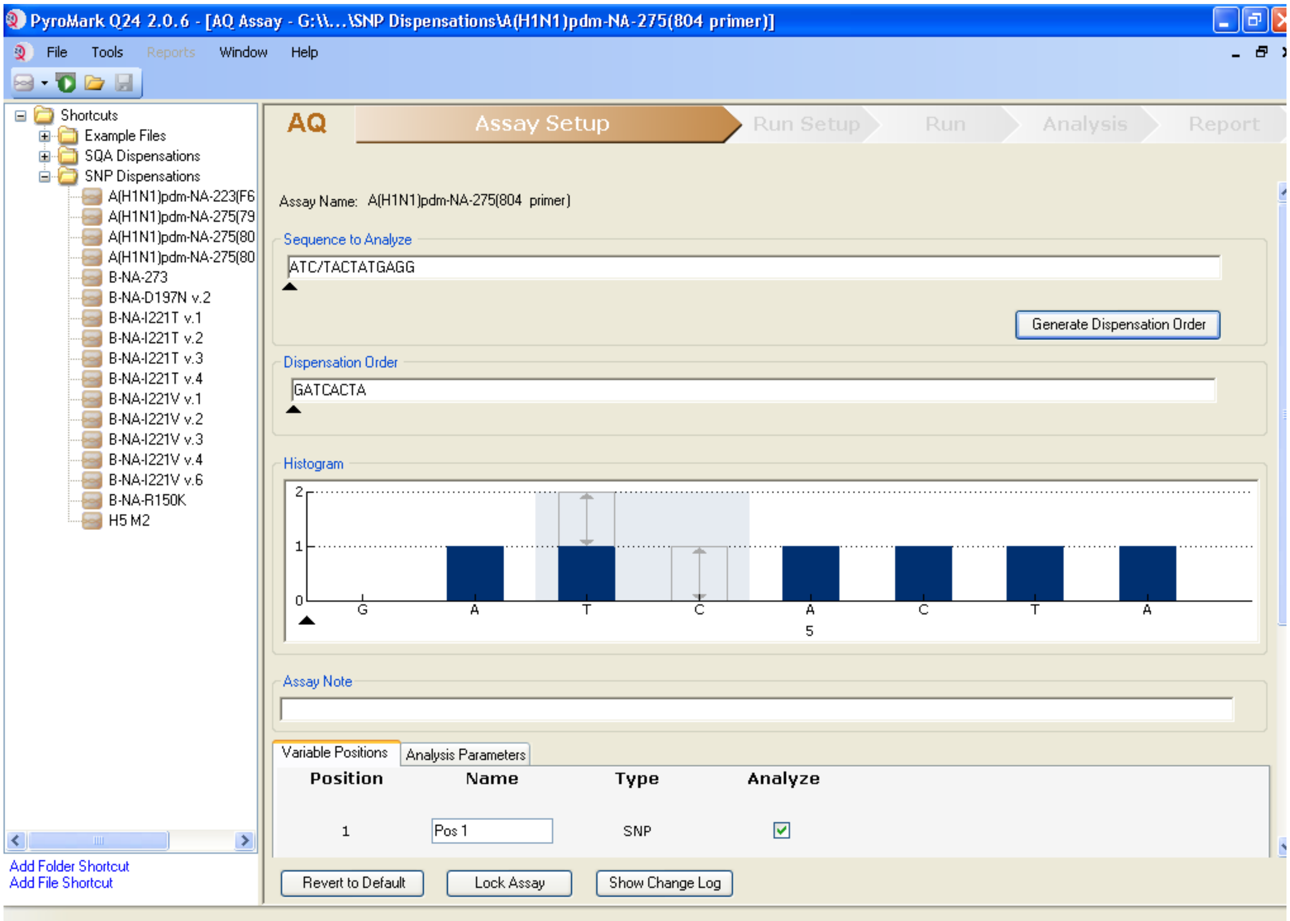
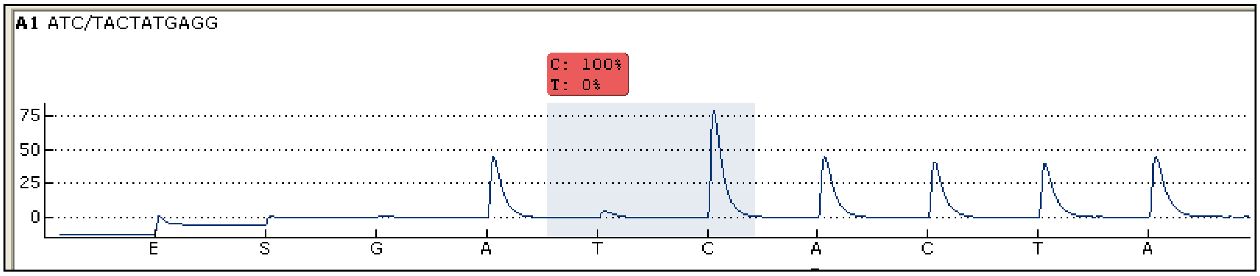


Figure 4. Example of Defining a SNP Entry.

A.



B.

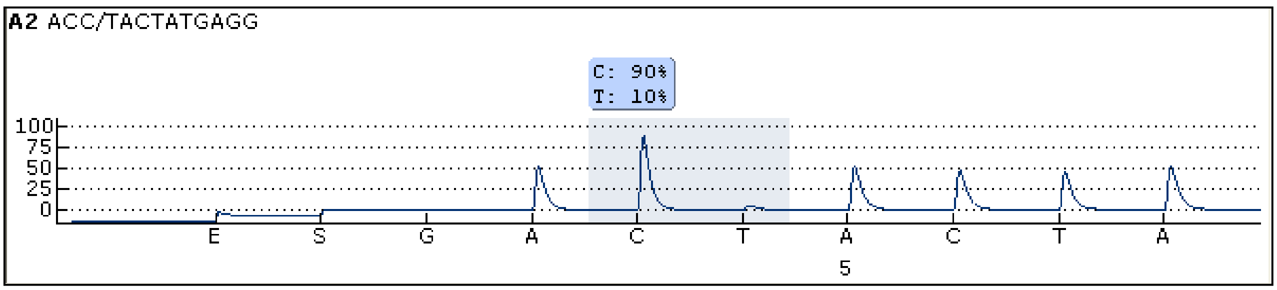


Figure 5. SNP Results for NA H275 with a Silent Mutation at Nucleic Acid Position 822 (T→C).

(A) Analysis using the dispensation generated for the major circulating virus variant (ATT/CATTA).

(B) Analysis using the dispensation generated for the silent virus variant (ACT/CATTA).

E	**A**	**S**
T		**C**
	G	

Label Should Face User

Figure 6. PyroMark™ Q24 Cartridge Setup.

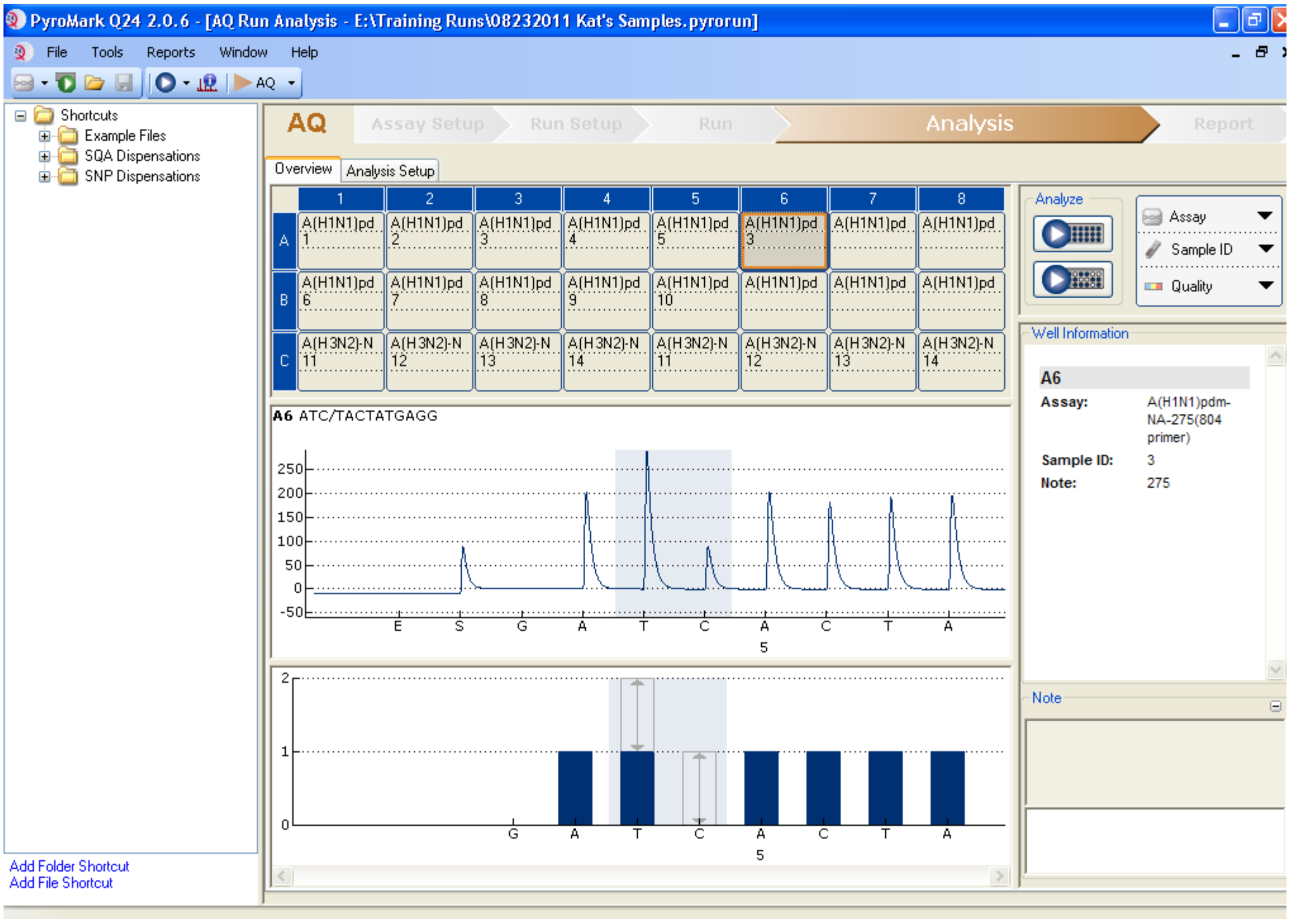


Figure 7. Example of a Completed SNP Run.

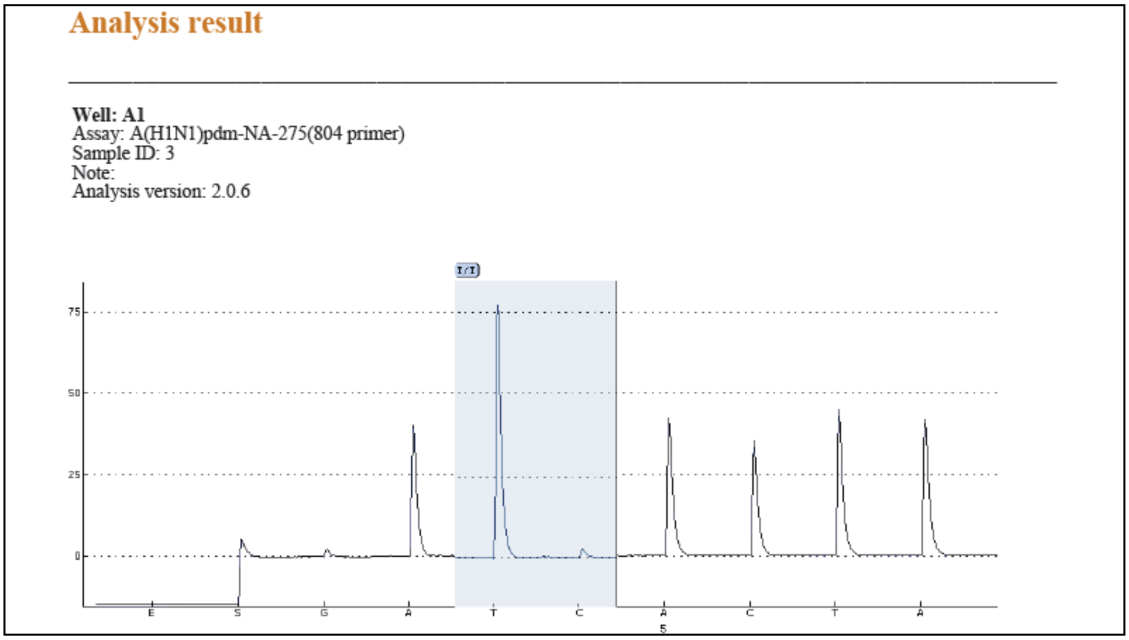


Figure 8. Example of a Report in SNP Mode.

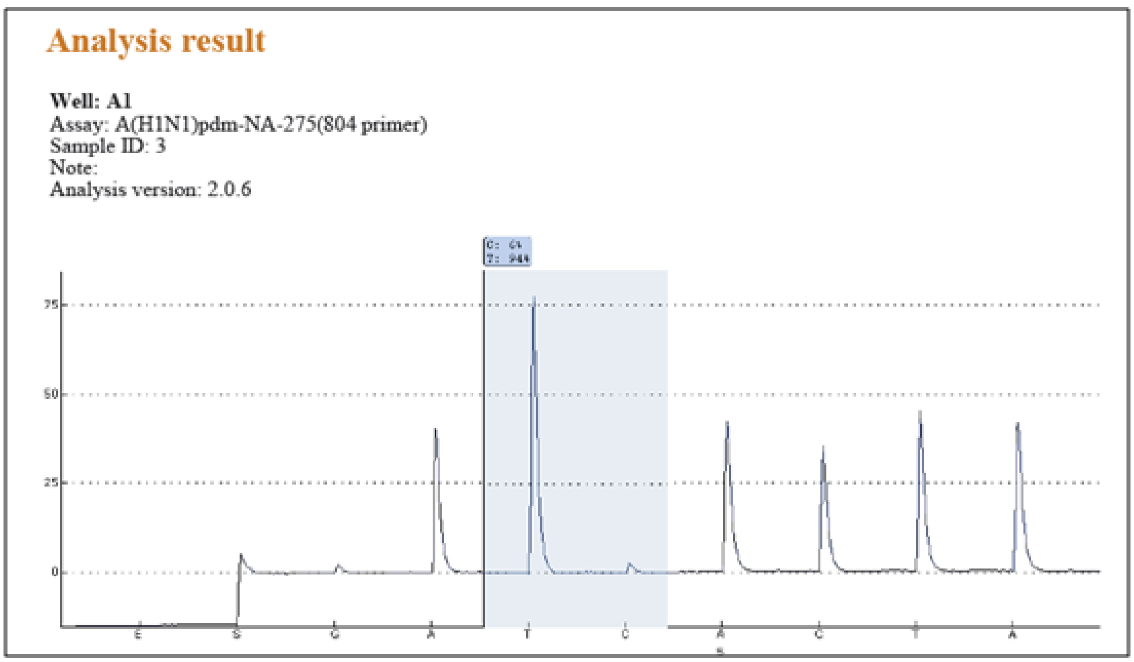


Figure 9. Example of a Report in AQ Mode.

VIII. Reference Material

NOTES: This document may not be used as a standalone document. This document is provided for Research Use Only. These protocols are not intended to be used for commercial development or for-profit testing.

Please do not distribute these procedures to other laboratories or commercial entities.

This additional document to the pyrosequencing protocol is for the detection of the H→Y substitution at position 275 in the NA of A(H1N1)pdm09 influenza viruses. These RT-PCR and pyrosequencing primers will generate sequences for pre-pandemic A(H1N1) viruses as well. The specific single nucleotide changes that are associated with the amino acid substitution H275Y in the NA are: H(CAC)→Y(TAC) for the A(H1N1)pdm09 or H(CAT) →Y(TAT) for the pre-pandemic A(H1N1). The pyrograms and the IdentiFire™ library provided in this section contains sequences for both A(H1N1)pdm09 and A(H1N1) viruses, so their sequences may be distinguished.

1 Reference Viruses

Below are examples of reference viruses carrying histidine (H) or tyrosine (Y) at position 275, which may be used in the NA-275 pyrosequencing assay:

Reference	Subtype	Variant
A/California/07/2009	A(H1N1)pdm09	H275
A/Washington/29/2009	A(H1N1)pdm09	H275Y
A/North Carolina/39/2009	A(H1N1)pdm09	H275Y
A/Washington/10/2008	H1N1	H275
A/Florida/21/2008	H1N1	H275Y

2 Primers

Table 1. RT-PCR Primers

Primer	Sequence 5'→ 3'	Concentration
Forward(sw-N1-F780)	GGG GAA GAT TGT YAA ATC AGT YGA	20 µM
Reverse(sw-N1-R1273-biot)	Biot-CWA CCC AGA ARC AAG GYC TTA TG	20 µM

Table 2. Sequencing Primer for amino acid at position 275 in the NA

Primer	Sequence 5'→ 3'	Concentration
Sequence(sw-N1-275-F804)	GYT GAA TGC MCC TAA TT	100 µM

3 RT-PCR Product

20 µl of RT-PCR product should be added to the respective wells of the plate containing the binding buffer solution.

4 **Target Entry Name:** A(H1N1)pdm09-NA-275

5 **Dispensation Order for SQA:** 5(CATG)

"CATGCATGCATGCATGCATG" should be visible under the expanded dispensation order window.

6 IdentiFire™ Library for Y275 Detection in Pandemic and Pre-pandemic Influenza H1N1 Viruses.

6.1 In the library (below), a few examples of possible sequences are provided. Examples: A(H1N1)pdm09-wildtype (H275) means the virus is a 2009 pandemic A(H1N1) carrying histidine at position 275; A(H1N1)pdm09-variant (Y275) means the virus is a 2009 pandemic A(H1N1) carrying the Y275 substitution; etc.

6.2 The below library also includes additional sequences containing silent mutations. For example, A(H1N1)pdm09-wildtype (H275)-T822C with a silent mutation at nucleotide 822 and pre-pandemic H1N1-wildtype (H275) with silent mutations for amino acids 279 and 280.

>A(H1N1)pdm09-wildtype (H275)
AT**CAC**TATGAGGAATGCTCC

>A(H1N1)pdm09-variant (H275Y)
AT**TAC**TATGAGGAATGCTCC

>A(H1N1)pdm09-wildtype (H275)-T822C
AC**CAC**TATGAGGAATGCTCC

>A(H1N1)pdm09-variant (H275Y)-T822C
AC**TAC**TATGAGGAATGCTCC

>Pre-pandemic H1N1-wildtype (H275)
TT**CAT**TATGAGGAATGCTCT

>Pre-pandemic H1N1-wildtype (H275)
TT**CAT**TATGAGGAATGTTCC

>Pre-pandemic H1N1-variant (H275Y)
TT**TAT**TATGAGGAATGCTCT

>Pre-pandemic H1N1-variant (H275Y)
TT**TAT**TATGAGGAATGTTCT

>Pre-pandemic H1N1-variant (H275Y)
TT**TAT**TATGAGGAATGTTCC

IX. References

Deyde V.M. and L.V. Gubareva. Influenza genome analysis using pyrosequencing method: current applications for a moving target. Expert Review of Molecular Diagnostics. 2009; 9(5): 493-509.

Deyde V.M., T. Nguyen, R.A. Bright, A. Balish, B. Shu, S. Lindstrom, A. I. Klimov, and L. V. Gubareva. Detection of molecular markers of antiviral resistance in influenza A(H5N1) viruses using a pyrosequencing method. Antimicrob Agents Chemother. 2009; 53(3): 1039-1047.

Deyde V.M., M. Okomo-Adhiambo, T. G. Sheu, T. R. Wallis, A. Fry, N. Dharan, A. I. Klimov, and L. V. Gubareva. Pyrosequencing as a tool to detect molecular markers of resistance to neuraminidase inhibitors in seasonal influenza A viruses. Antiviral Res. 2009; 81(1): 16-24.

Deyde V.M., T. G. Sheu, A. A. Trujillo, M. Okomo-Adhiambo, R. Garten, A. I. Klimov, and L V. Gubareva. Detection of molecular markers of drug resistance in 2009 pandemic influenza A (H1N1) viruses by pyrosequencing. Antimicrob Agents Chemother. 2010; 54(3): 1102–1110.

Sheu T.G., V.M. Deyde, R.J. Garten, A.I. Klimov, L.V. Gubareva. Detection of antiviral resistance and genetic lineage markers in influenza B virus neuraminidase using pyrosequencing. Antiviral Research. 2010; 85(2): 354-360.

If you have a technical question about this SOP, you may contact the CDC Molecular Epidemiology Team/Influenza Division at fluantiviral@cdc.gov.